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Caffeic Acid Metabolism by Bacteria of the Human Gastrointestinal Tract

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The metabolism of caffeic acid, previously shown to be carried out by the intestinal microbiota of man and experimental animals, has now been examined in a number of bacteria isolated from human feces. It has been found that of the 12 organisms isolated none has the ability to catalyze more than one reaction of the series leading from caffeic acid to either *m*-hydroxyphenylpropionic acid or 4-ethylcatechol.

Present knowledge of the microbial metabolism of caffeic acid can be summarized by the transformations shown in Fig. 1 (4, 14, 15; A. N. Booth and R. T. Williams, Biochem. J. Abstr., p. 66, 1963). Reactions supporting this scheme have been demonstrated in mixed flora derived from intestinal contents of several animal species (4, 14; A. N. Booth and R. T. Williams, Biochem. J. Abstr., p. 66, 1963). The conversions of caffeic acid either to dihydrocaffeic acid or to ethylcatechol are the only reactions of the scheme to be identified with a specific organism, *Lactobacillus pastorianus* var. *quinicus* isolated from cider (15).

The metabolism of caffeic acid, a component of the vegetable matter in the normal diet (9), has also been studied in man and experimental animals. When administered to man or rats, some additional transformations take place (e.g., methylation or conjugation with glycine) before the metabolites of caffeic acid are excreted in the urine (2, 3; E. N. F. Shaw et al., Proc. 5th Int. Congr. Biochem., p. 427, 1962). Nevertheless, the transformations of Fig. 1 as demonstrated in the intestinal microflora are the basis for caffeic acid metabolism in the whole organism. This assumption is supported by the observation that oral administration of neomycin, known to suppress the growth of intestinal bacteria, markedly diminishes the urinary excretion of compounds derived from caffeic acid (2; J. Dayman and J. B. Jepson, Biochem. J. Abstr., p. 11, 1969; E. N. F. Shaw et al., Proc. 5th Int. Congr. Biochem., p. 427, 1962).

To identify the specific microorganisms that might be responsible for the metabolism of caffeic acid in man, a number of different organisms normally found in the gastrointestinal tract have been tested for their ability to carry out the transformations of Fig. 1. Surprisingly, none of the individual bacteria tested had the ability to catalyze more than one reaction of caffeic acid metabolism that is summarized in Fig. 1.

MATERIALS AND METHODS

Bacteria. Bacteria used in this study are representatives of species that can be found in human feces. We thank Charles Zierdt who provided isolates 1 to 7. Other bacteria were isolated in pure culture from feces (normal volunteer) by using liquid media and agar plates whose composition is summarized in Table 1. Incubation conditions for these isolations are described below.

Materials. *m*-Hydroxyphenylpropionic acid was prepared by catalytic hydrogenation (palladium on charcoal) of *m*-hydroxycinnamic acid (6); the product was recrystallized from water, T_m, 109 to 110 C (literature value, 110 C; reference 14). Ethylveratrole, obtained by the reduction of 3,4-dimethoxyacetophenone (12), was demethylated (7) to yield 4-ethylcatechol. 4-Vinylcatechol was obtained by the microbial decarboxylation of caffeic acid under conditions which were a large-scale modification of those described in Table 2. When all the caffeic acid had been metabolized, the growth medium containing 4-vinylcatechol was sterilized by filtration and a sample was added to fresh growth medium for the study of other reactions. Compounds other than those mentioned above were obtained from commercial sources (Aldrich Chemical Co., Milwaukee; K & K Laboratories, Plainview, N. Y.; or Sigma Chemical Co., St. Louis). The identity of each of the metabolites used in the study was confirmed by mass spectrometry.

Incubation conditions. The metabolism of a compound was tested by adding it to the growth medium (10 ml) at a concentration of 0.5 mg/ml. The basic growth medium used was Thiol Broth (Difco) except for bacteria 10 to 12 for which Beef (Difco) was used. An inoculum (0.25 ml of a culture of less than 48 hr)

was added to the reaction mixture and the incubation was continued for 1 week at 37 C under anaerobic conditions (Gas Pak Anaerobic jars and H_2-CO_2 generator, BBL, Cockeysville, Md). Incubation mixtures from which either the bacterial inoculum or the compound to be tested was omitted served as controls.

Analysis of incubation products. At the end of the incubation, the growth medium was clarified by centrifugation and a preliminary assessment of metabolic transformation in comparison with the controls was obtained by gas-liquid chromatography (GLC). A sample of the incubation mixture (1 ml) was acidified to *p*H 1 by the addition of 5 N HCl and extracted three times with an equal volume of ether. The ether extracts were pooled, the ether was removed by evaporation at 38 C, and the trimethylsilyl ethers or esters were formed of the material in the residue (10). This material was then analyzed by GLC [6 ft (ca. 1.8 m) 3% OV 17 column, temperature programming between 100 and 250 C]. Identification of the products was based on GLC retention time and GLC-mass spectometry (LKB model 9000) with a 1% OV 17 column.

Quantification of metabolites. Catechols in the growth medium were quantified (1) using a Klett-Summerson Colorimeter, no. 56 filter, with pyrocatechol as a standard. For this procedure the catechols were separated from the catechol acids by adjusting the growth medium to *p*H 7.0 and extracting it three times with equal volumes of ether. The pooled ether extracts were then washed with water, dried over Na_2SO_4, and evaporated at 38 C, and the catechol content was measured. This colorimetric test was also used to quantify caffeic or dihydrocaffeic acid if there was no indication of metabolism to another compound containing the catechol group. Under these circumstances, ether extraction was not necessary and the colorimetric test was performed on the growth medium. In other instances, quantification of reactants and products was obtained by GLC. By the use of GLC, it was possible to detect the appearance of any of the products of Fig. 1 in an amount that would indicate a conversion of 1% of the starting material.

RESULTS

Transformation of caffeic acid by mixed cultures of feces. In an initial series of experiments, a small sample of human feces was used to inoculate incubation mixtures, each containing one of the compounds of Fig. 1; the reaction was allowed to continue as described above for periods up to 1 week. Under these conditions, evidence for transformation was detected only with caffeic acid, dihydrocaffeic acid, and 4-vinylcatechol. This finding is in accord with previous studies of cecal contents in rats where no metabolism of caffeic beyond *m*-hydroxyphenylpropionic acid or 4-ethylcatechol was detected (14).

Isolation of bacteria from feces. After these preliminary experiments on caffeic acid metabolism by mixed cultures, it was decided to study this pathway in cultures of individual organisms. Isolates 1 to 7 were obtained from patients at the

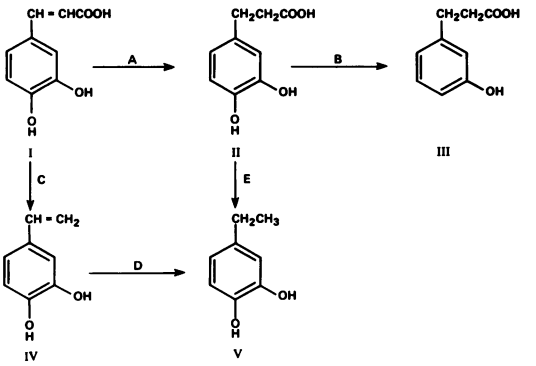

FIG. 1. *Metabolism of caffeic acid. Caffeic acid (I) is reduced to dihydrocaffeic acid [3,4-dihydroxyphenylpropionic acid (II)], which is dehydroxylated to m-hydroxyphenylpropionic acid (III). In another pathway, caffeic acid can be decarboxylated to yield 4-vinylcatechol (IV) which is reduced to 4-ethylcatechol (V). Reaction E, the decarboxylation of dihydrocaffeic acid is included for completeness, but there is no evidence for the occurrence of this reaction in this study.*

National Institutes of Health Clinical Center by using standard techniques of clinical microbiology. Caffeic acid or its metabolites, therefore, played no role in the selection of these bacteria. This contrasted to the selection of organisms 8 to 13 which were isolated in the presence of caffeic acid or one of its metabolites.

Transformation of caffeic acid and its metabolites by individual bacteria. To test the part played by each organism of Table 1 in the transformations of Fig. 1, each culture was allowed to react with either caffeic acid, dihydrocaffeic acid, or 4-vinylcatechol in the manner described above. The gas-liquid chromatograms of the reaction mixture and the control lacking the test compound were then compared. Any unique metabolites which appeared during the incubation were quantified by comparing the loss of test compound relative to the noninoculated control as well as by quantifying the appearance of any new compound. The metabolic transformations that occurred and their extent are summarized in Table 2. The total recovery of reactant and products was good in each case except for reactions C and D where the somewhat reduced recovery might be attributed to the lability of the catechols. In all incubations other than those listed in Table 2, no new compounds appeared and the recovery of the test compound was at least 90%; under these circumstances, it was considered that the microorganism was incapable of metabolizing the test compound.

From Table 2 it can be seen that *Peptostreptococcus* sp. and one of the two strains of *Clostridium perfringens* are capable of reducing caf-

feic acid (reaction A). Each of the other reactions is carried out either by one of the organisms isolated in pure culture or one of the mixed cultures. No single organism or the pair, 11 and 12, carries out more than one reaction of Fig. 1.

TABLE 1. *Identity and isolation conditions of bacteria*

No.	Identity of bacteria (genus)	Isolation medium[a]
1	*Bacteroides fragilis*	
2	*Clostridium perfringens*	
3	*Corynebacterium acnes*	
4	*Peptococcus* sp.[b]	
5	*Peptostreptococcus* sp.	
6	*Veillonella parvula*[b]	
7	*Fusobacterium* sp.	
8	*Clostridium perfringens*	Thio + caffeic acid
9	*Streptococcus fecium*[c]	Thio + caffeic acid
10	*S. fecalis*[c]	Beef + caffeic acid
11	*Escherichia coli*	Beef + dihydrocaffeic acid
12	*S. fecalis* var. *liquefaciens*[c]	Beef + dihydrocaffeic acid
13	Mixed culture	Thio + vinylcatechol

[a] Abbreviations: Thio, Thiol Broth (Difco); and Beef, beef prepared according to the *Difco Manual* (Difco Laboratories, Detroit). Where indicated, caffeic acid or its metabolites were added to the standard medium at a concentration of 0.5 mg/ml. The isolation medium for bacteria 1 to 7 is not listed since these organisms were obtained from stock cultures.

[b] Organisms 4 and 6 grew poorly in either Thio or Beef.

[c] The species determination of the Group D streptococci was based on fermentation differences for sorbitol, arabinose, arginine, and pyruvate as well as differential growth at 50 C and in tellurite (5).

The dehydroxylation of dihydrocaffeic acid (reaction B) requires a mixed culture of *Escherichia coli* (no. 11) and *Streptococcus fecalis* var. *liquifaciens* (no. 12); no reaction was detectable in an incubation with either organism alone. However, when grown in pure culture under conditions listed in Table 1, each organism grew rather poorly in comparison with the growth of the mixed culture. Better growth of the individual organisms was achieved on other media whose composition included dihydrocaffeic acid; but suspensions of cells grown under these conditions did not catalyze the dehydroxylation reaction even when both cell types were incubated together.

The microorganism(s) responsible for the reduction of 4-vinylcatechol (reaction D) were not identified. They were present in successive transfers into liquid medium of an inoculum of human feces, but they apparently required conditions which prevented their isolation in pure culture from plates under the conditions used.

Identification of products. The identity of compounds isolated from the growth media was established by their characteristics on GLC-mass spectrometry in comparison with the appropriate authentic compounds. An authentic standard of 4-vinylcatechol was not available, but the mass spectrum was consistent with this structure; in addition, the isolated compound had the same ultraviolet spectral absorbance maximum (258 nm) as the literature value (8). Further confirmation of the identity of 4-vinylcatechol was obtained by its reduction to 4-ethylcatechol both

TABLE 2. *Metabolism of caffeic acid and its products by the bacteria*[a]

Bacterial identity classification	No.	Metabolic reaction[b]	Compound tested		Reaction product	
			Name	Remaining at 1 week (%)	Name	Conversion (%)
Peptostreptococcus sp.	5	A	Caffeic acid	90	Dihydrocaffeic acid	6
Clostridium perfringens	8	A	Caffeic acid	90	Dihydrocaffeic acid	10
Escherichia coli and *Streptococcos fecalis* var. *liquifaciens*[c]	11, 12	B	Dihydrocaffeic acid	50	*m*-Hydroxyphenylpropionic acid	45
S. fecium	9	C	Caffeic acid	0	4-Vinylcatechol	50
Impure culture[d]		D	4-Vinylcatechol	0	4-Ethylcatechol	70

[a] All incubations were in Thiol Broth (Difco) except that designated B which was in Beef (Difco); other conditions are described in Materials and Methods.

[b] Metabolic reactions are lettered in accordance with Fig. 1.

[c] Both bacteria were necessary to catalyze reaction B.

[d] Unlike the individual organisms (or pair of organisms) described above, the impure culture was capable of other reactions with caffeic acid and its metabolites.

by catalytic hydrogenation (palladium on charcoal) and microbiologically. The ultraviolet absorbance maximum at 278 nm found for 4-ethylcatechol is consistent with the literature value (279 nm, reference 14).

DISCUSSION

It is striking that none of the organisms used in this study is capable of carrying out more than one reaction of the overall pathway of caffeic acid metabolism. This observation suggests that caffeic acid metabolism, previously studied by using unfractionated intestinal contents, may represent the combined capability of various members of the indigenous flora rather than the ability of a single organism. Of course this hypothesis must be considered against the background of the selection of organisms used for this study. Caffeic acid or one of its metabolites was present in the medium used to isolate organisms 8 to 13. The possible influence of these additions on the selection procedure is difficult to assess. Perhaps if the basic media were less rich, organisms isolated in the presence of caffeic acid or its metabolites might have been capable of more complete utilization of caffeic acid. On the other hand, organisms 1 to 7 whose isolation was independent of caffeic acid also showed limited ability to transform caffeic acid or its metabolites.

Another possible limitation of this study is the arbitrary selection of media used to examine caffeic acid metabolism. Growth of the organisms under other conditions might have led to different capability in regard to their metabolism of the test compounds. Evidence that this may be the case can be seen in studies directed at elucidating the basis for the collaboration of *S. fecalis* var. *liquifaciens* (no. 12) and *E. coli* (no. 11) in carrying out the dehydroxylation of dihydrocaffeic acid. When the two organisms were grown independently under other growth conditions, there was no evidence that they could dehydroxylate dihydrocaffeic acid even when resting cells of both organisms were incubated with the compound. Thus growth conditions obviously play a role in the metabolic capability of these organisms.

At least two possible mechanisms might be suggested to explain the requirement for two organisms in the dehydroxylation reaction. One might be the influence of one organism in providing the growth conditions necessary for the second to produce the enzyme(s) which catalyze the reaction. A second explanation is that the dehydroxylation occurs in two steps, each catalyzed by an enzyme present in one of the organisms. For example, it could be postulated that dihydrocaffeic acid is reduced to the 3,4-dihydrodiol of phenylpropionic acid which is then dehydrated in a second reaction to yield *m*-hydroxyphenylpropionic acid. In such a scheme, the accumulation of the proposed intermediate might be prevented by thermodynamic factors such as the oxidation-reduction potential of the medium. Under such circumstances, it would be necessary that both organisms be present simultaneously to establish favorable energy coupling for the overall reaction. Although the data do not allow a decision between the two explanations, both emphasize the need for collaboration of the two organisms.

A similar need for collaboration between organisms occurs in the reduction of 4-vinylcatechol. Presumably the presence of other microorganism(s) is required to provide cultural conditions necessary for the growth of the organism(s) which carry out this reaction. Our failure to isolate the responsible organism may be a manifestation of the extremely anaerobic conditions necessary for the cultivation of many of the bacteria of the gastrointestinal tract (11, 13).

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